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Inflammopharmacology

ORIGINAL ARTICLE

# Immunomodulatory potential of nanocurcumin-based formulation

**Mahendra Kumar Trivedi[1] · Sambhu Charan Mondal[2] · Mayank Gangwar[2] · Snehasis Jana[2]**

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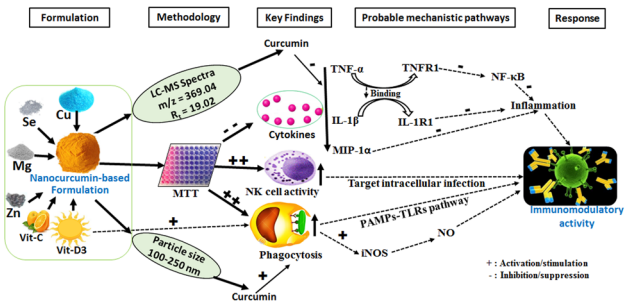
**Abstract** Vitamins, minerals, and nanocurcumin play a substantial role in various nutraceutical/pharmaceutical formulations that are widely used in therapeutics, cosmetics, and dietary supplements. The current study aimed to investigate the comparative in vitro immunomodulatory effect of a novel nanocurcumin-based formulation with curcumin in LPS-induced cytokine expression, NK cells' activity, and phagocytosis. The proinflammatory cytokines (TNF-α, IL-1β, and MIP-1α) and NK cells' activity were measured in cell supernatants using ELISA assay; however, phagocytosis activity was performed using colorimetric analysis. The chemical characterization of novel nanocurcumin-based formulation using LC–MS ($R_t$ 19.02 min) and mass spectra analysis ($m/z$ 369.04) confirmed the presence of the curcumin in highest peak concentration. MTT assay in three tested cell-lines showed that the formulation was found non-toxic at all the tested concentrations. The expression of TNF-α, IL-1β, and MIP-1α in splenocytes was significantly ($p \leq 0.001$) inhibited. Besides, the NK cells' activity and phagocytosis (macrophage) were increased significantly ($p \leq 0.001$). Overall, the promising results of this study indicated the significant immunomodulatory effect of nanocurcumin-based formulation compared to the curcumin, which could be used against various inflammatory disorders such as allergy, asthma, autoimmune diseases, coeliac disease, inflammatory bowel disease, etc.

*Graphical Abstract*




**Keywords** Nanocurcumin · Immunomodulation · Pro-inflammatory cytokines · Natural killer cells · Phagocytosis · Liquid chromatography-mass spectrometry

## Introduction

The immune system plays a crucial role in maintaining the proper health and keeps us safe from infection and diseases. It fights against the disease causing pathogens or any other agents that enter the body and prevent it from causing any damage (Janeway et al. 2001). Many herbal extracts either per se or in combination with minerals have the significant activities on inflammatory cytokines (Burns et al. 2010). Herbal medicinal preparations can favorably regulate the whole immune system (Spelman et al. 2006). As a consequence, the finding of the lead compound with lower toxicity and higher immunomodulatory activity is of great interest. In recent years, medicinal plants have been used for thousands of years in clinical practice which provide a vast source of pharmaceutical material for the development of

✉ Snehasis Jana
jana@trivedisrl.com

[1] Trivedi Global, Inc., Henderson, USA

[2] Trivedi Science Research Laboratory Pvt. Ltd., Bhopal, Madhya Pradesh, India

effective drugs that offer some unique advantages with low toxicity profiles (Wong 2001; Serkova et al. 1996). A herbomineral formulation containing ashwagandha along with essential minerals, such as magnesium, zinc, and selenium showed wide array of therapeutic activity which was reported to be a suitable candidate for multi drug targeting for several diseases (Trivedi et al. 2017a). In continuation, with our research on herbomineral-based formulation for various clinical benefits such as prevention and treatment of different type of diseases, overall growth improvement, strengthening of the body immunity, etc., authors formulated a novel formulation composing of nanocurcumin based on its significant potential along with vitamins like ascorbic acid, cholecalciferol, and several mineral salts viz. zinc chloride, magnesium gluconate hydrate, ferrous sulfate, sodium selenate, and copper chloride. The novel nanocurcumin-based formulation has been reported with significant immunomodulatory action in animal model (Trivedi et al. 2017b).

The role of curcumin has been well defined by Yadav et al. (2005), for immunomodulation, anti-inflammatory, antioxidant, and chemopreventive properties, while nanocurcumin was reported to have better bioavailability than curcumin. Sankar et al. (2013) demonstrated the immunomodulatory effects of nanocurcumin, and they found that the magnitude of the immune response of nanocurcumin was better than free curcumin at the equivalent dose level. Besides, nanocurcumin formulation enriched with vitamins and minerals has not yet been studied that can be used in order to replenish the immune system. However, the rationale behind selecting the combination of divalent cations (viz. $Zn^{2+}$, $Mg^{2+}$, $Fe^{2+}$, and $Cu^{2+}$) is the formation of chelate with nanocurcumin, which results in better stability and solubility of the formulation (Zebib et al. 2010). NK cells are important effector cells of the innate immune system. Activation of NK cells results in their cytotoxic activity against locally attached target cells and leads to the secretion of cytokines. The cytotoxic activity of NK cells occurs through the granule secretory pathway, which is responsible for the clearance of viral and malignantly transformed cells from the body (Pardo et al. 2009). Reduction of the natural killer (NK) cells functions after exposure to toxic chemicals that lead to suppression of immune response. Vitamin C enhanced NK cells' activity in patients by tenfold (Heuser and Vojdani 1997).

Minerals like zinc, copper (Stabel and Spears 1989), and magnesium have potential role in cytokine expression through induction or inhibiting the activation of NF-κB (Kruse-Jarres 1989; Zhou et al. 2010; Sugimoto et al. 2012) in inflammatory condition. Supplementation of ferrous sulfate can restore bone marrow homeostasis. It also plays a significant role in the proliferation and telomerase activity of lymphocytes (Yang et al. 2006). Selenium is an essential trace element that plays an important role in protecting cells from oxidative stress and thus reduces the risk of cardiomyopathy, cancer, and immune disorders in humans (Rayman 2000). Phagocytic process in immune cells is responsible for maintenance of homeostasis and diseases (Gordon 2016). For instance, in metabolic conditions, i.e., diabetes, the phagocytic activity is reduced, while improved phagocytosis can be achieved after insulin treatment (Lecube et al. 2011). Considering the above facts and information, authors aimed to illustrate the lipopolysaccharide (LPS)-induced immunomodulatory activity of the nanocurcumin-based test formulation and curcumin in three different cell lines (splenocytes, Yac-1, and macrophage) with the estimation of defined pro-inflammatory cytokine expression (TNF-α, IL-1β, and MIP-1α), NK cells' activity, and phagocytosis.

## Materials and methods

### Chemicals and reagents

MTT, LPS, L-glutamine, RPMI-1640, penicillin, HEPES, streptomycin, 2-mercaptoethanol, cholecalciferol (vitamin D3), copper (II) chloride, and iron (II) sulfate heptahydrate were purchased from Sigma Chemical Co. St. Louis, MO., USA. Rapamycin was purchased from Clearsynth Labs Ltd., Mumbai, India. FBS was procured from GIBCO, USA. Nanocurcumin (>95%) and curcumin (99%) were procured from Sanat product Ltd., Delhi, India, and Qualikems Fine Chem Pvt. Ltd., Delhi, India, respectively. Zinc chloride and magnesium (II) gluconate hydrate were procured from TCI, Japan. Sodium selenate and ascorbic acid (vitamin C) were procured from Alfa Aesar, USA. ELISA kits for all cytokines such as TNF-α, MIP-1α, and IL-1β were purchased from R&D Systems, USA. Yac-1 (Mouse lymphoma cell line) and RAW 264.7 (Mouse Macrophage cell line) were procured from National Centre for Cell Sciences (NCCS), Pune, India. All other chemicals used in the experiment were of analytical grade available in India.

### Composition and preparation of the nanocurcumin-based test formulation

The novel nanocurcumin-based formulation was composed of nanocurcumin (200 mg/mL) along with five trace metals and two vitamins viz. zinc chloride (0.397 mg/mL), ferrous sulfate (0.569 mg/mL), sodium selenate (0.00137 μg/mL), copper (II) chloride (0.121 mg/mL), magnesium gluconate (6.5 mg/mL), vitamin C (0.572 mg/mL), and cholecalciferol (0.0191 mg/mL). The required quantity of all the above individual ingredients

was mixed together with the total formulation concentration 208 mg/mL after considering the salt correction factor (molecular weight of salt/molecular weight of element) and solubility. The above formulation was vortexed to achieve a homogenous solution, which was considered as 100% stock solution. The above stock solution was further diluted in serum-free medium (SFM) to obtain a range of concentrations (in % v/v) for subsequent treatment.

### Characterization of test formulation

The particle size of nanocurcumin in the test formulation was analyzed using Malvern Mastersizer 3000, UK by following the wet method (Priyadarsini 2014). Nanocurcumin-based formulation was characterized by liquid chromatography-mass spectrometry (LC–MS) using LC-Dionex Ultimate 3000 and Thermo Scientific TSQ Endura Mass Spectrometer, USA. The analytical column reversed phase C18 (Zorbax SB, 100 × 4.6 mm, 3.5 µm) was used for this study. Mobile phases were 2 mM ammonium format and 0.5% formic acid in water (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.6 mL/min. The column temperature was kept constant at 40°C. The injected sample volume was 10 µL and the total run time was 35 min. The eluate was introduced directly into the electro spray ionization source, which was operated in positive ion mode of a triple quadrupole mass spectrometer.

### Experimental design

The experiment was designed into five groups. Group 1 contained the defined cells without LPS, denoted as normal control (NC). Group 2 contained the defined cells in DMSO along with LPS and denoted as vehicle control (VC) group. Group 3 and 4 were defined as positive control of rapamycin (RAP) and curcumin (CUR), respectively, along with LPS. Group 5 was denoted as the nanocurcumin-based test formulation group at various concentration that included respective cells with LPS.

### Animal care and housing

C57BL/6 male mouse (8 weeks old) was purchased from Ms. Vivo Bio Tech Ltd., Hyderabad, India. Rodent laboratory chow diet and drinking tap water were provided ad libitum and maintained under controlled conditions: a temperature of 22 ± 3 °C, humidity of 30–70% and a 12-h light/12-h dark cycle. All the animals were carefully handled humanely with due regard for their welfare. Animals were procured from the registered animal house facility (Reg. no. 64/PO/br/s/99/CPCSEA), Dabur Research Foundation (DRF) for experiment of animals with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India. The animals were procured using Animal Ethics Committee approved protocol (IAEC/36/391 dated 25.07.2016) and the husbandry conditions maintained as per CPCSEA recommendations.

### Cell culture and maintenance

The mouse was used for the isolation of splenocytes as per standard protocol (Trivedi et al. 2017a). The LPS (0.5 µg/mL) induced splenocyte cell cultures were grown for 48 h at 37 °C in a humidified $CO_2$ incubator (5% $CO_2$) (Małaczewska 2014). The single-cell suspension of splenocytes was co-incubated with the mouse lymphoma cells (Yac-1), which were plated at a density of $2 \times 10^4$ cells/well in 96-well culture plates in RPMI medium containing 10% FBS for the estimation of natural killer (NK) cells' activity. The LPS (0.5 µg/mL) induced splenocyte cell cultures were grown for 24 h at 37 °C in a humidified $CO_2$ incubator (5% $CO_2$) (Jin and Kruth 2016). The macrophage ($5 \times 10^4$ cells per well) cells were grown in 24-well culture plates using RPMI-1640 medium supplemented with 10% FBS, 100 µg/mL of streptomycin, and 100 units/mL of penicillin for phagocytosis activity. The LPS (0.5 µg/mL) induced macrophage cell cultures were grown as per Yac-1 culture condition (Berridge and Tan 1993). The respective vehicle control kept in the assay was DMSO with LPS.

### MTT assay

Cytotoxicity was determined by exposing cells (splenocytes, Yac-1, and macrophage) to different concentrations of test formulation in RPMI. The respective vehicle control kept in the assay was DMSO with LPS. The number of viable cells was estimated based on the conversion of MTT to formazan dye using a mitochondrial enzyme. The effect of the test formulation on cell viability of splenocytes was determined with the help of Eq. 1:

$$\% \text{ Cell viability} = (100 - \% \text{ cytotoxicity}) \quad (1)$$

where % cytotoxicity = {(O.D. of control cells − O.D. of cells treated with test formulation)/OD of control cells} × 100 (Berridge et al. 1996).

### Cytokine assay

The effect of the test formulation on the production of TNF-α, IL-1β, and MIP-1α was measured by ELISA method in culture supernatants using a Biotek reader (SIAFRT/Synergy HT multimode reader). For the estimation of TNF-α, IL-1β, and MIP-1α, LPS (0.5 µg/mL) induced splenocytes were exposed to the test formulation at

selected non-toxic concentration. After 48 h of incubation, supernatants were analyzed for the secreted levels of cytokines as per manufacturer's instructions (Keustermans et al. 2013; Stockerta et al. 2012; Leng et al. 2008).

### Natural killer (NK) cells assay

The effect of the test formulation on NK cell activity was performed in culture supernatants (mouse splenocytes) by ELISA method using Biotek reader (SIAFRT/Synergy HT multimode reader) according to the manufacturer's instructions (Lee et al. 2014; Holt et al. 2012). This assay was used for the quantitative detection of the killing of Yac-1 cells by NK cells (splenocytes) in the mouse lymphoma cell line (Yac-1). For the estimation of NK-cells' activity, LPS (0.5 μg/mL) induced Yac-1 cell-line was exposed to the test formulation at selected non-toxic concentration (1.3–9.9 μg/mL). The resultant modulatory effect of test formulation on NK cells' activity in the presence of LPS was determined with the help of measured optical density of Yac-1 cells alone or in combination with NK cells. Further, increase in % NK cell activity was measured using the following Eq. 2:

$$\begin{aligned} \%\ \text{NK cells activity} &= (\%\ \text{NK cells activity in LPS} \\ &+ \%\ \text{Test formulation treated cells}) \\ &- \%\ \text{NK cells activity in control cells} \end{aligned} \quad (2)$$

### Phagocytosis assay

The effect of the test formulation on phagocytosis assay was measured in culture supernatants using CytoSelect™ 96-well phagocytosis assay kit (Zymosan, Colorimetric Format) as per manufacturer's instructions (Cell Biolabs Inc. USA). This assay was used for quantitative colorimetric detection of engulfed prelabeled zymosan particles by a mouse macrophages cell line (RAW264.7). For the estimation of the extent of phagocytosis in LPS (0.5 μg/mL) induced macrophage, the cells were exposed to the test formulation at selected non-toxic concentrations. After 24 h of incubation, supernatant was analyzed for the assessment of phagocytosis assay using colorimetric method as per manufacturer's instructions (Yan et al. 2015; Higa et al. 1998). The resultant modulatory effect of test formulation-mediated phagocytosis in the presence of LPS was determined with the help of measured optical density of LPS-induced macrophages cell line in control group and test formulation group.

### Statistical analysis

All the data analysis was performed with SigmaPlot Statistical Software (Version 11.0). The difference between the mean values (in triplicate) was assessed for statistical difference using one-way analysis of variance (ANOVA). $p < 0.05$ was considered as statistically significant. The results are shown as the mean ± standard error of mean (SEM).

## Results

### Characterization of test formulation

The LC–MS spectrum of the test formulation was performed, and the results are presented in Fig. 1a, b. The total ion chromatogram (TIC) analysis showed $R_t$ value as 19.02 min that indicated the presence of curcumin with highest peak area %. The analysis suggested that the concentration of curcumin was greater than 90% as compared to the other constituents present in the test formulation. The mass spectrum of the test formulation showed the base peak at *m/z* value 369.04 $[M+H]^+$ that confirmed the molecular mass of curcumin, which was well corroborated with the reported literature (Ramalingam and Ko 2014). Besides, the particle size analysis showed that the particle size was in the range of 100–250 nm. The nano-sized curcumin particles in the test formulation could be attributed to larger surface area, which might increase the dissolution and absorption and result in enhanced biological activities with improved efficacy, solubility, and bioavailability.

### Assessment of cytokine production

#### *Cells viability by MTT in splenocytes*

The cell viability of rapamycin (RAP) was increased by 30.3, 21.3, and 13.8% at 0.1, 1, and 10 nM, respectively as compared to the normal control (NC). Further, curcumin (CUR) showed increased cell viability by 75.68, 70.55, and 58.94% at 7.4, 10, and 25 μg/mL, respectively, as compared to the NC. Besides, the nanocurcumin-based test formulation stimulated the cell proliferation by 10.88, 9.31, and 8.89% at 4.9, 6.7, and 9.9 μg/mL, respectively, as compared to the NC. Overall, all the tested concentrations showed more than 98% cell viability, which was selected for subsequent cytokines' estimation.

#### *Expression of TNF-α*

The level of TNF-α in the normal control (NC) group was 53.9 ± 6.23 μg/mL and in the LPS-induced VC group it was observed as 380.4 ± 12.74 μg/mL. The results of the nanocurcumin-based test formulation demonstrated a significant ($p \leq 0.001$) suppression of TNF-α level by 16.79 and 22.40% at 4.9 and 9.9 μg/mL, respectively, compared

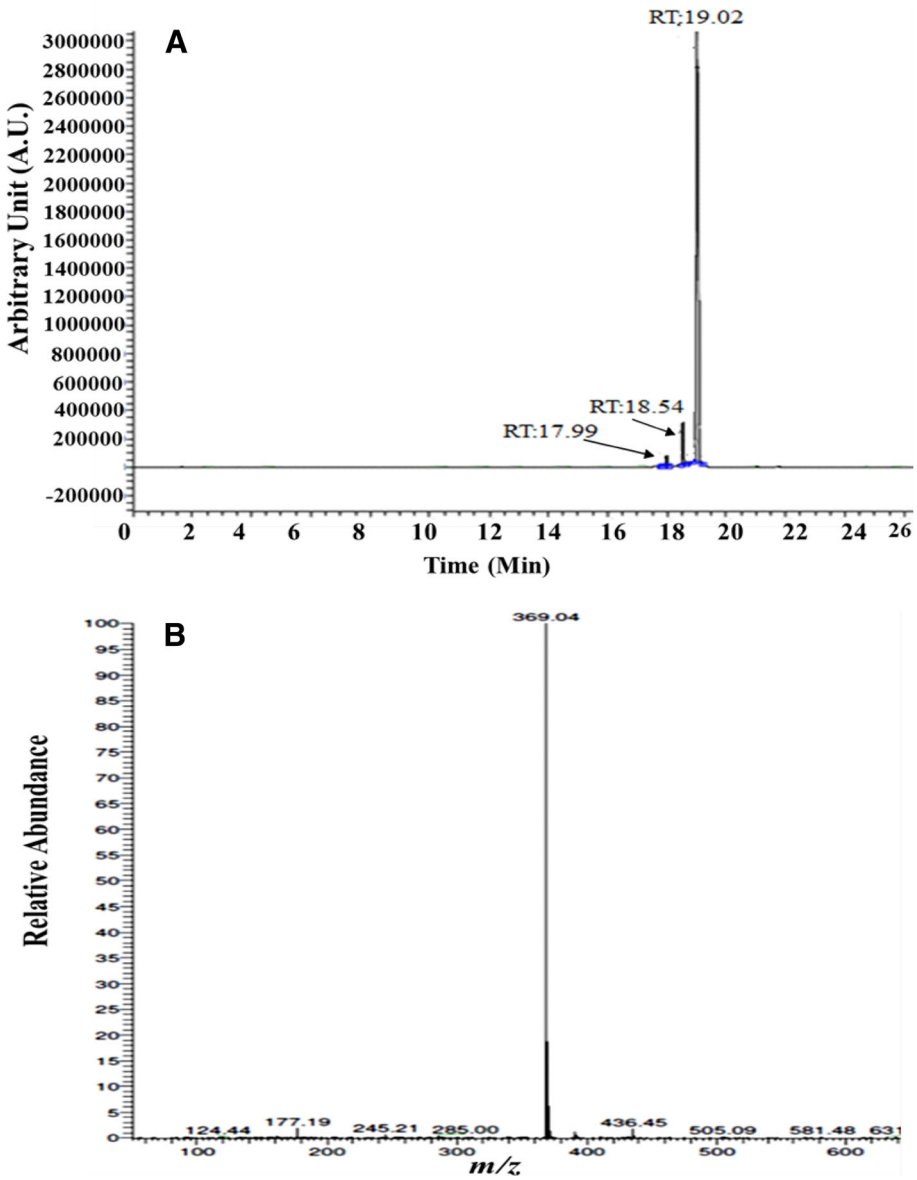



**Fig. 1** Characterization of nanocurcumin-based test formulation. **a** Total ion chromatogram (TIC) of the formulation and **b** mass spectra ($m/z$ = 369.04) at $R_t$ = 19.02 indicating curcumin

with the LPS-induced VC group (Fig. 2). RAP group showed significant ($p \leq 0.001$) suppression of TNF-α by 26.46, 26.72, and 33.54% at 0.1, 1, and 10 nM, respectively, as compared to the VC. Moreover, the level of TNF-α was decreased by 13.15% in the CUR at 10 μg/mL, compared to the VC. Overall, the nanocurcumin-based test formulation revealed more suppression of TNF-α expression than CUR treated group.

*Expression of IL-1β*

The level of IL-1β in the NC group was 20.76 ± 3.03 pg/mL and it was significantly increased by 120.76% in the VC group (45.83 ± 3.83 pg/mL) after induction with LPS. The nanocurcumin-based test formulation showed a significant ($p \leq 0.001$) inhibition of IL-1β secretion by 21.75, 27.73, and 44.43% at 4.9, 6.7, and 9.9 μg/mL as compared with the LPS-induced VC group (Fig. 3). Besides, RAP showed significant reduction of IL-1β secretion by 14.05 and 29.41% ($p \leq 0.001$) at 1 and 10 nM, respectively, compared to the VC. Further, the level of IL-1β secretion was suppressed by 16.31 and 34.10% in the CUR group at 7.4 and 10 μg/mL, respectively, as compared to the VC.

*Expression of MIP-1α*

The level of MIP-1α in the NC group was 100.51 ± 12.81 pg/mL and in the VC group 1337.44 ± 36.59 pg/mL after induction with LPS. The nanocurcumin-based test formulation showed significant ($p \leq 0.001$) inhibition of MIP-1α secretion by 17.56 and 26.72% at the tested concentration, i.e., at 4.9 and 9.9 μg/

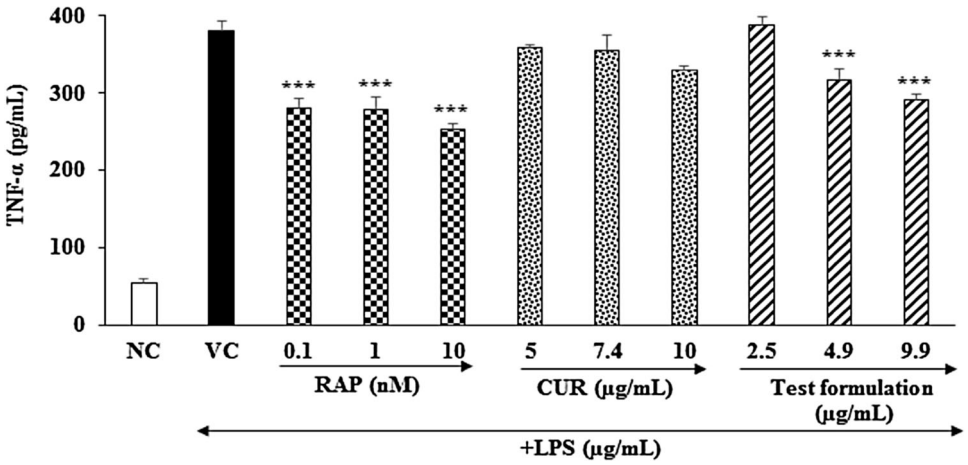



**Fig. 2** Effect of the nanocurcumin-based test formulation on TNF-α secretion in LPS-mediated splenocyte cells by ELISA method using Biotek reader at 450 nm measured after 48 h exposure. *NC* normal control, *LPS* lipopolysaccharide, *VC* vehicle control, *RAP* rapamycin, *CUR* curcumin, *Test formulation* nanocurcumin-based formulation. ***$p \leq 0.001$ vs VC (using one-way ANOVA)

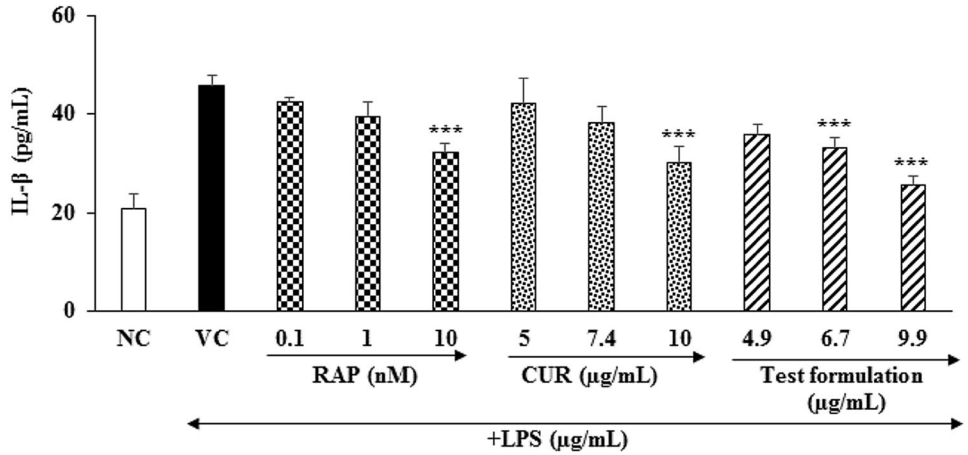



**Fig. 3** Concentration-dependent inhibition of IL-1β expression of nanocurcumin-based test formulation in LPS-mediated splenocyte cells by ELISA method using Biotek reader at 450 nm. *NC* normal control, *LPS* lipopolysaccharide, *VC* vehicle control, *RAP* rapamycin, *CUR* curcumin, *Test formulation* nanocurcumin-based formulation. ***$p \leq 0.001$ vs VC (using one-way ANOVA)

mL, respectively, as compared to the LPS-induced VC group (Fig. 4). The level of MIP-1α was significantly inhibited by 30.12, 40.40, and 40.27% at 0.1, 1, and 10 nM, respectively, in the RAP group as compared to the VC. MIP-1α was reduced by 18.47% at 10 µg/mL in the CUR group as compared to the VC. Hence, the test formulation showed better suppression of MIP-1α even at a low concentrations compared with the CUR.

## Assessment of natural killer cells' activity

The natural killer (NK) cells' activity is shown in Fig. 6. The nanocurcumin-based test formulation showed significant activation of NK cells' activity by 20.99, 36.54% ($p \leq 0.001$), and 47.51% at 4.9, 6.7, and 9.9 µg/mL, respectively, as compared to the vehicle control. The NK cells' activity was significantly increased by 10.74 and 28.76% at 1 and 10 nM, respectively, in the RAP group as compared to the VC. Further, CUR data exhibited an elevation of NK cells' activity by 10.52 and 20.56% at 5 and 10 µg/mL, respectively, as compared to the VC (Fig. 5). Overall, the nanocurcumin-based test formulation showed better NK cells' activity as compared to the curcumin.

## Assessment of phagocytosis

### *Cell viability by MTT assay in RAW 264.7 (mouse macrophage cell-line)*

The normal macrophage cells and vehicle control groups showed 100 and 94.5% cell viability, respectively. All the concentrations of the tested formulation showed >77% cell viability in mouse macrophage cell line as compared to the vehicle control group. The percentage cell viability was increased in some of the tested concentrations with respect to the normal control, which might be due to the proliferation in cell culture (Fig. 6). Moreover, the nanocurcumin-based test formulation showed more viable cells with respect to the CUR.

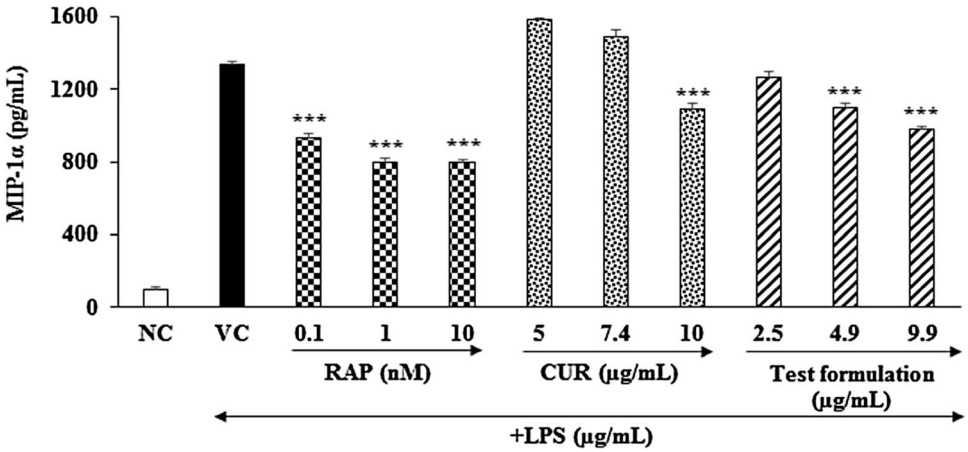



**Fig. 4** Effect of nanocurcumin-based test formulation on the expression of MIP-1α in LPS-mediated splenocytes. ***$p \leq 0.001$ vs VC (Using one-way ANOVA). *NC* normal control, *LPS* lipopolysaccharide, *VC* vehicle control, *RAP* rapamycin, *CUR* curcumin, *Test formulation* nanocurcumin-based formulation

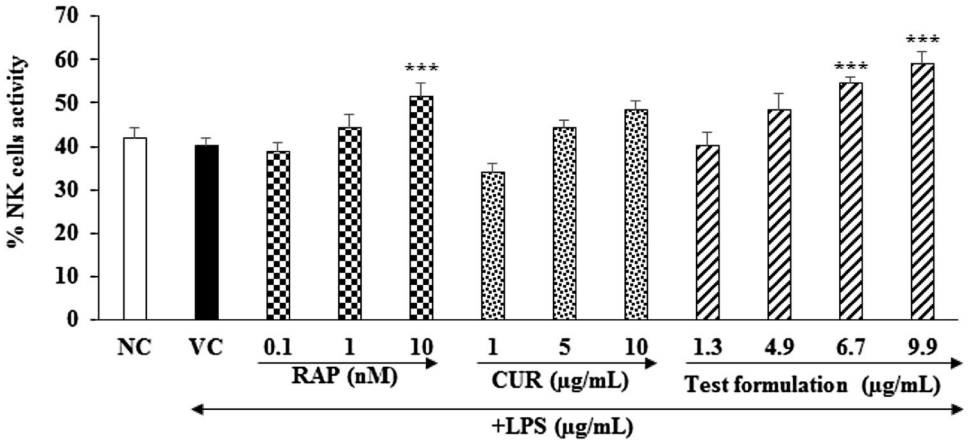



**Fig. 5** Effect of the nanocurcumin-based test formulation on natural killer cells' activity in LPS-mediated splenocytes co-incubated with Yac-1. ***$p \leq 0.001$ *vs* VC (Using one-way ANOVA). *NC* normal control, *LPS* lipopolysaccharide, *VC* vehicle control, *RAP* rapamycin, *CUR* curcumin, *Test formulation* nanocurcumin-based formulation

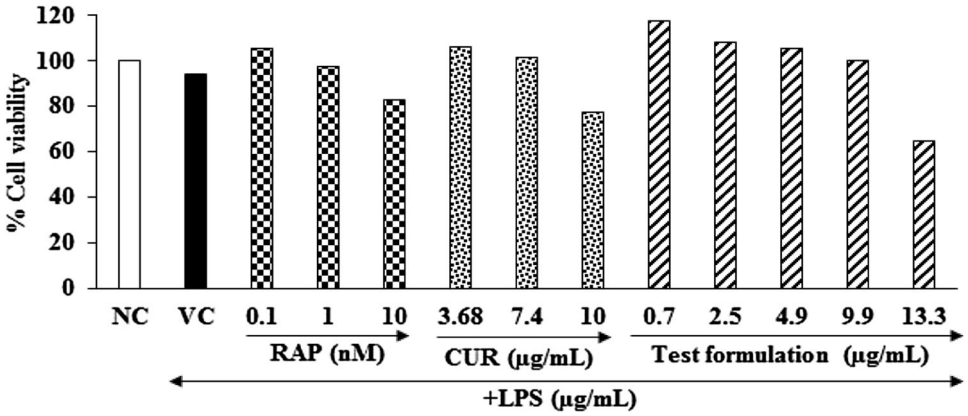



**Fig. 6** Measurement of cell viability by MTT assay in mouse macrophage cell line (RAW264.7). *NC* normal control, *LPS* lipopolysaccharide, *VC* vehicle control, *RAP* rapamycin, *CUR* curcumin, *Test formulation* nanocurcumin-based formulation

*Phagocytosis*

The maximum percentage of phagocytosis in the CUR group was 19.74% at 1.8 μg/mL; however, in the nanocurcumin-based test formulation group it showed higher value as 22.20% at 1.3 μg/mL. Furthermore, the nanocurcumin-based test formulation exhibited significant ($p \leq 0.001$) increase in the phagocytosis activity by 15.11 and 21.83% at 2.5 and 6.7 μg/mL, respectively, as compared to the VC. Besides, the RAP group showed significant increased phagocytosis activity by 19.97 and 33.45% ($p \leq 0.001$) at 1 and 10 nM, respectively. From the study results, it was concluded that the nanocurcumin-

based test formulation adjunct with vitamins and minerals showed better response than CUR per se (Fig. 7).

## Discussion

Curcumin possesses therapeutic efficacy against a wide variety of diseases, and is also found to be safe at high doses. Unfortunately, it has poor solubility, stability, and rapid metabolism; hence the bioavailability of curcumin is the major concern (Dutta and Ikiki 2013). In this perspective, authors focus a nanocurcumin-based formulation in this experiment for the assessment of immune response in three different cell lines. Nanocurcumin was used in combination with divalent cations such as $Zn^{2+}$, $Mg^{2+}$, $Fe^{2+}$, and $Cu^{2+}$ that are supposed to form a chelate complex with a keto-enol group of curcumin and thus enhanced the stability as well as the absorption of the nanocurcumin-based formulation. Furthermore, with added vitamins like ascorbic acid and cholecalciferol it also enhanced the free-radical scavenging activity.

Vitamin D3 can modulate the key elements of innate immunity and has been used for the prevention and treatment of pneumococcus-induced inflammation (Olliver et al. 2013). Besides, immune modulating potential of cholecalciferol (vitamin D3) as a modulator of calcium homeostasis has been described predominantly (Correale et al. 2009). Zinc deficiency enhanced the expression of cytokines like IL-1β, IL-2, IL-6, and TNF-α. Besides, after supplementation of zinc, the level of cytokines was restored in a dose-dependent manner through induction or inhibiting the activation of NF-κB (Kruse-Jarres 1989; Zhou et al. 2010). Magnesium salts can reduce the level of TNF-α and effectiveness in those at risk for inflammation. It reversibly regulates the cytokine production via transcriptional regulation by reducing NF-κB activation (Sugimoto et al. 2012). Copper plays an important role in the maintenance of immunocompetence. Deficiency of copper results in decreased humoral and cell-mediated, as well as nonspecific immune function (Stabel and Spears 1989). Cytokines are the key factors in acute and chronic inflammation. Inflammation is characterized by an interplay between pro- and anti-inflammatory cytokines (Cavaillon 2001). Cytokines are critical mediators of both the innate and the adaptive immune responses, which play an important role in immunomodulation. Inflammation plays a significant role in most of the diseases, including cancer (de Visser et al. 2006). Detection of the proinflammatory cytokines is important in both animal models and in human patients (Amsen et al. 2009) as immunity markers. The different types of immune cells such as dendritic cells, macrophages, and splenocytes play an important role in order to stop the acute/chronic inflammation and retrieve a steady-state strategy through the secretion of immuno-modulating cytokines (Mosmann and Sad 1996). These immune cells have been reported to be useful as cellular models for in vitro studies. Therefore, authors used murine splenocytes to assess the in vitro immunomodulatory effect by measuring the proinflammatory cytokines' and NK cells' activity of the test formulation. Formulating new products that have the ability to improve the overall health by reducing inflammation is essential because of the potential for long-term effectiveness, decrease toxicities, and lower costs.

The MTT test is used for the evaluation of the metabolic activity of a mitochondrial enzyme. This test is widely used for the evaluation of the toxicity of any test item/formulation in vitro (Zhang and An 2007). Based on the cell viability using MTT assay showed the novel nanocurcumin-based test formulation was found to be safe at the tested concentration, while the cell viability was reported as >100% (approx.) in mouse splenocytes (Fig. 2). Hence, all the tested concentrations were selected within this range for the estimation of cytokines, NK cells' activity, and

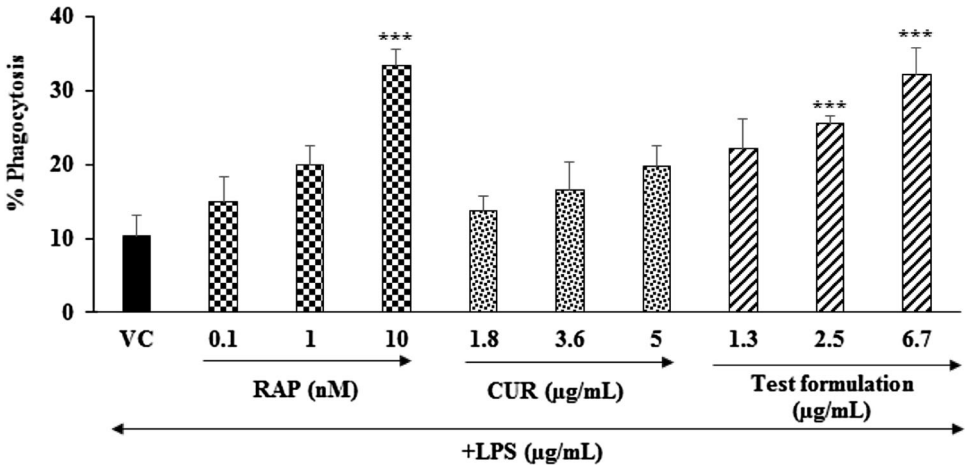



**Fig. 7** Effect of the nanocurcumin-based test formulation on the percent change of phagocytosis in LPS-mediated mouse macrophage cell line. *LPS* Lipopolysaccharide, *VC* vehicle control, *RAP* rapamycin, *CUR* curcumin, *Test formulation* nanocurcumin-based formulation. ***$p \leq 0.001$ vs VC (Using one-way ANOVA)

phagocytosis. It can be concluded that the test formulation showed an increase in the cell viability at the specified concentrations with respect to both normal and vehicle control groups. In this experiment, the level of various proinflammatory cytokines was significantly suppressed at different concentrations by the newly developed novel test formulation. This suppression might be due to the presence of either nanocurcumin or it could be due to the presence of complex with several macro and micro-elements and vitamins in the test formulation. Numerous scientific reports and clinical trials had evidenced the immunomodulatory activity of minerals and vitamins (Mazumder et al. 2012; Ren et al. 2012; Aranow 2011). The trace elements viz. zinc, magnesium, selenium, copper, manganese, etc. also play an important role in immunomodulation, as they have a strong interaction with the immune system (Bahi and Necib 2014). Through a receptor in the immune cells, vitamin D increases the phagocytic activity of macrophages and NK cells (Radović et al. 2012).

TNF-α plays a central role in inflammation, immune modulation, and lymphocyte activation (Sibi and Rabina 2016). Our results showed a significant ($p \leq 0.001$) down-regulation of TNF-α expression at the tested concentrations. It is evident that the formulation could be used against various inflammatory disorders by regulating the expression of TNF-α. Down-regulation of IL-1β expression during infections with respect to the immunological and inflammatory functions is well established (Singh et al. 2007). The experimental results exhibited a significant ($p \leq 0.001$) inhibition of the expression of IL-1β at three concentrations as compared to the vehicle control group. The results suggested that higher concentrations showed better immunosuppressive activity with respect to the lower tested concentrations of the test formulation. TNF-α and IL-1β can act through binding with TNFR1 and IL-1R1 receptors, respectively, and activate the NF-κB and lead to inflammation (Russo and Polosa 2005; Edye et al. 2014). Thus, the nanocurcumin-based formulation exhibited anti-inflammatory activity through down-regulating the expression of TNF-α and IL-1β through NF-κB activation mechanism. MIP-1α plays an important role in mediating the acute inflammatory response in trauma-hemorrhage and reported that MIP-1α reduction could be beneficial in minimizing the inflammatory responses in several diseases (Hsieh et al. 2008). The data revealed that MIP-1α level was significantly ($p \leq 0.001$) suppressed after exposure of the novel formulation in splenocyte cells.

Natural killer (NK) cells are mainly found in a nonimmune cell population that has the ability to lyse certain types of NK-sensitive target cells to recognize and kill tumor cells in vitro. Activation of the NK cells leads to increased cytolytic and proliferative functions that mediate anti-tumor activity. Therefore, it is important for NK cells to mobilize from a resting state (Holt et al. 2012; Wright and Bonavida 1983). The results described that NK cells' activity was increased in the vehicle control group, which was due to LPS stimulation of LPS. Several scientific kinds of literatures well corroborated our findings. LPS has a pleiotropic effect on the immune system and thus activates macrophages, lymphocytes, and NK cells (Conti et al. 1991; Kanevskiy et al. 2013). Further, it was found that NK cells' activity was accelerated by the tested test formulation group at three tested concentrations out of four in a dose-depended manner. From the literature, it was observed that curcumin (CUR) enhanced the NK cells' activity by inducing the process of apoptosis (Fiala 2015) and anti-proliferative effects on various cancer cell lines (Khatwani et al. 2016). From our study results, it was found that the novel nanocurcumin-based test formulation showed better NK cells' activity as compared with the CUR group.

Phagocytosis is a cellular process that plays crucial role in the removal of dead or dying cells, tissue remodeling, and host defense against invading microbes (Mosser and Zhang 2011). Macrophages are mononuclear phagocytes present in the body responsible for the development, homeostasis and participate in innate and adaptive immune responses and wound healing (Zhang et al. 2008). The test formulation has been found to be effective for the significant improvement in the process of apoptosis by phagocytosis at the concentrations of 2.5 and 6.7 μg/mL. The immunomodulatory response through an increased level of phagocytosis had governed through numerous mechanistic pathways like activation of iNOS followed by the massive release of NO (Song et al. 2015) and PAMPs-TLRs pathway (Mogensen 2009). Altogether, experimental data showed that the novel nanocurcumin-based proprietary formulation remarkably down-regulated the expression of pro-inflammatory cytokines like TNF-α, MIP-1α, and IL-1β with improved natural killer cells' activity and phagocytosis.

This experiment investigated a novel nanocurcumin-based formulation adjunct with couple of vitamins and multiple minerals for its immunomodulatory activity. Due to the presence of divalent cations (viz. $Zn^{2+}$, $Mg^{2+}$, $Fe^{2+}$, and $Cu^{2+}$) in the formulation, absorption, as well as the stability of the nanocurcumin-based formulation, can be improved through the formation of chelate with a keto-enol group of curcumin. Besides, the presence of vitamins like ascorbic acid and cholecalciferol also aggravates free-radical scavenging activity. Overall, this novel nanocurcumin-based formulation would be helpful in the pharmaceutical industry with improved immunomodulatory activity than curcumin per se. Thus, the novel formulation could be useful as an anti-inflammatory product in immunocompromised patients as well as healthy human to improve the quality of life.

## Conclusion

The study results concluded that the novel nanocurcumin-based test formulation showed significant down-regulation of TNF-α, IL-1β, and MIP-1α expression as compared to the vehicle control group in mouse splenocyte cells. Additionally, the natural killer cells' activity and phagocytosis were improved significantly at higher concentrations. Overall, data indicated a significant reduction of proinflammatory cytokines and improved natural killer cells' activity and extent of phagocytosis upon exposure with the nanocurcumin-based test product on respective cell-lines. In conclusion, the nanocurcumin based-formulation adjunct with vitamins and minerals could be a better nutraceutical product than curcumin per se with various pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, and anti-carcinogenic. Together, the results of the present study demonstrated the improved potential of nanocurcumin-based formulation compared with curcumin in managing various types of inflammatory disorders.

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**Compliance with ethical standards**

**Conflict of interest** The authors report no conflicts of interest.

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